Mitochondrial function and toxicity: Role of B vitamins on the one-carbon transfer pathways

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Abstract

The B vitamins are water-soluble vitamins that are required as coenzymes for reactions essential for cellular function. This review focuses on the essential role of vitamins in maintaining the one-carbon transfer cycles. Folate and choline are believed to be central methyl donors required for mitochondrial protein and nucleic acid synthesis through their active forms, 5-methyltetrahydrofolate and betaine, respectively. Cobalamin (B12) may assist methyltetrahydrofolate in the synthesis of methionine, a cysteine source for glutathione biosynthesis. Pyridoxal, pyridoxine and pyridoxamine (B6) seem to be involved in the regeneration of tetrahydrofolate into the active methyl-bearing form and in glutathione biosynthesis from homocysteine. Other roles of these vitamins that are relevant to mitochondrial functions will also be discussed. However these roles for B vitamins in cell function are mostly theoretically based and still require verification at the cellular level. For instance it is still not known what B vitamins are depleted by xenobiotic toxins or which cellular targets, metabolic pathways or molecular toxic mechanisms are prevented by B vitamins. This review covers the current state of knowledge and suggests where this research field is heading so as to better understand the role vitamin Bs play in cellular function and intermediary metabolism as well as molecular, cellular and clinical consequences of vitamin deficiency. The current experimental and clinical evidence that supplementation alleviates deficiency symptoms as well as the effectiveness of vitamins as antioxidants will also be reviewed.

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1. Introduction

Until the folate fortification program was implemented in 1998, 25–50% of the US population was thought to be deficient in folate, while 5–10% of the population may have been deficient in vitamin B12 and 10% in vitamin B6 [1]. All three vitamins (Table 1) are required as cofactors or substrates for enzymes essential for cell function. One-carbon metabolism is important for protein and DNA methylation and also for the synthesis of nucleotides. The former reaction requires first the transfer of a methyl group to S-adenosylhomocysteine (SAH), forming the universal methyl donor S-adenosylmethionine (SAM), while the latter is dependent on one-carbon-bearing folate metabolites in thymidylate or purine synthesis. As both folates and choline can provide the one-carbon group in the reactions leading to SAM formation, the genetic risks associated with DNA misincorporation or mutation may
Table 1
Structures of B vitamins

<table>
<thead>
<tr>
<th>Type</th>
<th>Name</th>
<th>Chemical structure</th>
</tr>
</thead>
</table>
| B6   | Pyridoxal| ![Pyridoxal](image1)
|      | Pyridoxamine | ![Pyridoxamine](image2)
|      | Pyridoxine  | ![Pyridoxine](image3)
| B9   | Folate    | ![Folate](image4)
| B12  | Cobalamin | ![Cobalamin](image5)

This table lists the major dietary forms of the three B vitamins reported in this review.

be a greater risk for folate deficiency than that associated with protein or DNA hypomethylation.

For each of these water-soluble vitamins, we will consider the biochemical evidence relating to absorption, metabolism and the essential role of the bioactive form of the molecules, concentrating on one-carbon transfer pathways and other vitamin-dependent reactions relevant to mitochondrial functions. In another
issue in this issue, we described the role of other B vitamins in maintaining mitochondrial pathways of energy production [2]. Vitamins B6 and B12, but not folate, play a role in those pathways, the consequences of which will be discussed here. Current and potential biomarkers of vitamin deficiency will be described, followed by evidence for the therapeutic use of B vitamin supplementation. Clinical conditions associated with specific B vitamin deficiencies (proven or suggested) are listed in Table 2, based on data collected from Refs. [3,4] and recent publications (not cited here). The aim of this review is to better understand the relationship between mitochondrial dysfunction caused by vitamin deficiency and possible clinical outcomes. It is important to note that we are defining mitochondrial dysfunction as any direct or indirect change in the cellular functions that will affect reactions localized in the mitochondrial matrix or that have consequences on mitochondrial stability (e.g. mitochondrial membrane potential). This will include pathways ranging from energy metabolism (reviewed in this issue [2]) but also the antioxidant balance or more general mitochondria-based pathways.

### 2. Folates

Folate was identified in the late 1930s as the nutrient required to reduce anemia during pregnancy. Other possible folate associations have been described over the following years and are listed in Table 2.

#### 2.1. Folate delivery from the diet to mitochondrial enzymes

Folate was originally identified in yeast and liver. The vitamin is most abundant in unprocessed grains, oranges, dried beans, peas, eggs and green vegetables. Since 1998 processed grain products (flours, bread, cereals) have been fortified and have become a significant source of dietary folate. Fortification of foods was associated with a decrease in neural tube defects such as spina bifida [5] and possibly pediatric neuroblastoma [6]. Folate deficiency can result from an inadequate dietary folacin (folic acid) content, malnutrition or increased metabolic needs caused by pregnancy/metastatic cancer or antifolate chemotherapy, e.g. methotrexate.

Folates in the foods are mostly bound to proteins as polyglutamates that cannot be absorbed as such and must be hydrolyzed by proteases first to the monoglutamate forms before absorption in the small intestine. Absorption occurs primarily in the proximal jejenum via the reduced folate carrier of the brush-border. The carrier activity is maximal at pH 6.3, and is mediated partly through folate−/OH− system, and partly by H+ co-transporters. Reduced monoglutamylfolate in the portal blood is taken up by the reduced folate carrier in the liver sinusoidal membrane, mediated by H+ co-transport. Other cells may use a folate binding protein (folate receptor) in the cell membrane and mitochondrial membrane for the uptake of folate and 5-methyltetrahydrofolate (CH3THF) [7]. Inside cells and mitochondria the folate monoglutamates are elongated to the poly-γ-glutamate derivatives, catalyzed by folyl poly-γ-glutamate synthetase [8]. This results in the retention and compartmentalization of folate cofactors in the cell and the mitochondria. Fig. 1 shows the various metabolic fates of folate proposed for the cytosol and mitochondria fractions of the cell. In the hepatocyte CH3THF and homocysteine are converted to tetrahydrofolate (THF) and methionine by a reaction catalyzed by methionine synthase (reaction 9, Fig. 1). Folate is also reduced to dihydrofolate (DFH) and THF using NADPH, catalyzed by dihydrofolate reductase (reaction 1, Fig. 1) [9]. Tetrahydrofolate enters the mitochondria and reacts with serine catalyzed by serine hydroxymethyl transferase (reaction 2, Fig. 1) to form

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**Table 2**

Clinical disorders associated with vitamin deficiencies

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Established condition of deficiency</th>
<th>Possible associations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate (B9)</td>
<td>Megaloblastic anemia / Neural tube defects, with perinatal exposure</td>
<td>Colon cancer / Cardiovascular disease / Pediatric leukemia / Pediatric neuroblastoma</td>
</tr>
<tr>
<td></td>
<td>Pernicious anemia (macrocystic anemia and subacute combined degeneration of the spinal tracts with polyneuritis)</td>
<td>Cardiovascular disease / Stroke / Impaired cognitive function / Cancerogenesis</td>
</tr>
<tr>
<td>Cobalamin (B12)</td>
<td></td>
<td>Osteoporosis / Neural tube defects / Multiple sclerosis</td>
</tr>
<tr>
<td>Pyridoxal (B6)</td>
<td>Epileptic seizures / Anemia / Renal failure</td>
<td>Cardiovascular disease / Colon cancer / Reduced cognition, depression / Alzheimer’s disease</td>
</tr>
</tbody>
</table>

This table is a list of clinical consequences of deficiency of folate, cobalamin or pyridoxal. The links between the clinical symptoms and vitamin deficiency were based on clinical measurements of vitamin levels in an affected population, regression of symptoms upon vitamin supplementation, or animal models. Conditions that have been established are listed on the left, and others that have been suggested are found on the right. These possible associations listed in the last column have not yet been proven in a rigorous manner.
glycine and 5,10-methenyltetrahydrofolate (CH$_2$THF). The latter is reduced to formate and THF, catalyzed by 10-formyltetrahydrofolate synthetase (reaction 5, Fig. 1). The unique transporter identified in the mitochondrial inner membrane that transports THF and other folates into the matrix has been named the mitochondrial folate transporter and has been sequenced [10].

2.2. Essential role of folate in mitochondrial and cellular function

Folates function as a family of cofactors that carry one-carbon (C1) units required for the synthesis of thymidylate, purines, and methionine, and required for other methylation reactions. Folate is essential for metabolic pathways involving cell growth, replication and the survival of cells in culture. Thirty to fifty percent of cellular folates are located in the mitochondria. It is generally thought that the mitochondrial folates are the primary actors of serine/glycine cycling, while the cytosolic folates are the primary location for thymidylate, purine and methionine synthesis. As shown in Fig. 1, the proper functioning of mammalian folate metabolism requires two separate independent compartments in the cell containing parallel but distinct folate metabolizing enzymes. The mitochondrial folate pool does not equilibrate with the cytosolic pool. Major folate metabolites found in the cytosol were THF and CH$_3$THF whereas in the mitochondria they were THF and formyltetrahydrofolate (CHO-THF).

In the mitochondria reduced folates are required for three functions: (1) the initiation of mitochon-
Fig. 2. Choline metabolism and choline-dependent methyl-transfer pathways. The figure is compiled from various sources and represents the choline catabolism pathway. Reactions are catalyzed by the following enzymes: (1), choline dehydrogenase; (2), betaine aldehyde dehydrogenase; (3), betaine: homocysteine S-methyl transferase; (4), dimethylglycine dehydrogenase; (5), sarcosine dehydrogenase; (6), serine hydroxymethyl transferase; (7), glycine cleavage system; (8), phosphoethanolamine methyl transferase/phospholipid-\(\text{N}\)-methyl transferase (need three cycles of PEA to PC); (9), phosphocholine phosphatase. Reaction 6 is the only step of this pathway that is reversible. Choline itself can however be formed from phosphate ethanolamine or phosphatidylethanolamine through methyl transfer from SAM (8). A number of metabolites in this pathway interact with other pathways. For example betaine feeds into homocysteine metabolism (as an equivalent to methionine synthase depicted in Fig. 1), while glycine and serine are being used in a large number of reactions, including methyl transfer to or from tetrahydrofolate (Fig. 1, reaction 2) or formation of glutathione (Fig. 3). Again NAD\(^+\) plays an important role in the pathway, and reactions 4, 5 and 7 use FAD as cofactor while reactions 6 and 7 require PLP as coenzyme.

In the cytosol the tetrahydrofolate metabolites carry C1 units in the form of formyl groups. These are used for the synthesis of purines and thymidylate required for cytosolic and mitochondrial ATP formation and DNA synthesis. As shown in Fig. 1, a CH\(_3\) group is transferred to homocysteine from CH\(_3\)THF via the formation of methylcobalamin intermediate. The reaction is catalyzed by methionine synthetase (reaction 9, Fig. 1) to form methionine. Methionine could then be used for cysteine synthesis required for GSH and protein synthesis as well as for the formation of SAM that serves as the one-carbon donor for the methylation of DNA, RNA, protein and xenobiotics. Although liver mitochondria lack methionine adenosyl transferase, SAM is readily transported into mitochondria via a passive-carrier-mediated system [13].

In the formate pathway most of the methylating enzymes are reversible including the terminal step catalyzed by 10-formyltetrahydrofolate synthetase
(reaction 5, Fig. 1) that forms formate, ATP and tetrahydrolfolate. The reverse of this step is a major detoxifying pathway for formate. Formate is converted to 10-formyl tetrahydrolfolate catalyzed by the synthetase and oxidation to CO$_2$ catalyzed by 10-formyltetrahydrolfolate dehydrogenase (reaction 7, Fig. 1) to reform tetrahydrolfolate.

2.3. Folate deficiency

Folate status is commonly assessed as plasma or erythrocyte folate. Deficiency can also be quantitated by an increase in plasma levels of homocysteine (hyperhomocysteinemia) and glycine (hyperglycinemia), although homocysteinemia, as detailed later in this review, can also be induced by a number of other deficiencies and disorders.

Folate-dependent pathways and homocysteine provide a first approach to assessing deficiency. Folate deficient diets in rats decreased serum folate and increased homocysteine [14], as well as cystathionine, choline metabolism intermediates (glycine, serine, N,N-dimethylglycine, and N-methylglycine), but decreased tryptophan metabolites in NAD synthesis (kynurenine, kynurenic acid, quinolinic acid) [15]. Folate and choline deficiency decreased nicotinamide adenine dinucleotide (NAD) and NADP levels, even when niacin was present in the diet [16].

Folate-dependent pathways and DNA damage provide still another class of markers. Folate deficiency increased reticulocyte micronuclei in mice and rats, suggesting that it resulted in chromosomal instability and chromosomal breaks [17]. In folate-methyl deficient rats, lesion-containing hepatic DNA was less efficiently methylated than lesion-free DNA and an increase in DNA strand breaks preceded DNA hypomethylation. This suggests that DNA lesions may be required for the disruption of normal DNA methylation [18]. Folate deficiency leads to chromosome break via uridine incorporation in DNA instead of thymidine [14]. During the DNA repair process induced by this misincorporation, single strand breaks are formed. If two uridine bases have been introduced on the two DNA strands in close proximity, a double strand break that is more difficult to repair can be formed and can lead to a chromosome break or sister chromatid exchange [19].

Folate deficiency in the brain can be assessed in several additional ways. In mice folate deficiency, and increased homocysteine levels, decreased the number of proliferating cells in the hippocampus and induced DNA damage and apoptosis in rat hippocampal neurons in association with mitochondrial dysfunction and oxidative stress [20]. Perinatal folate deficiency in mice also resulted in an increase in congenital defects including neural tube defects with behavioral consequences [21].

Increased plasma homocysteine levels are a risk factor for cardiovascular disease, stroke and neurodegenerative disorders in humans. Homocysteine–thiolactone is a reactive plasma homocysteine metabolite formed from homocysteine by most cells as a consequence of the error-editing reactions of aminoacyl tRNA synthetases which ensure that homocysteine cannot complete the protein biosynthetic pathway. Folate facilitates the conversion of homocysteine to methionine and thus indirectly prevents thiolactone formation. Once the thiolactone is formed it binds covalently to protein lysine residues. In the blood it is mostly bound to albumin or hemoglobin. In human endothelial cells, however, it can induce a caspase independent apoptotic death that is initiated by a collapse of the mitochondrial membrane potential [22]. It is not clear whether thiolactone formation within cells reaches a high enough concentration to produce the pathological changes associated with cardiovascular or neutodegenerative disease.

2.4. Prevention of oxidative stress and mitochondrial toxicity by folates

Folic acid can act as a direct antioxidant and scavenger molecule. In vitro pulse radiolysis studies have shown that folic acid can scavenge and repair peroxyl, hydroxyl and thiol radicals through oxidation of the purine hydroxyl groups [23]. Furthermore it can prevent microsomal lipid peroxidation despite its water solubility [23]. In vivo $^{2,14}$C-folic acid is oxidatively cleaved at the C$_3$–N$_{10}$ bond to form p-acetamidobenzoic acid and p-acetamidobenzoyl-L-glutamate which are excreted in the urine [24]. The mechanism suggested is the oxidation of the tetrahydrofolate by ROS to quinonoid dihydrofolate which undergoes an oxidative cleavage to yield 7,8-dihydroppterin and p-aminobenzoyl-L-glutamate. Ethanol or drugs increasing ROS increased these urinary products in hamsters. The increased folate requirement by alcoholics could involve such a mechanism [24]. High dose folate supplementation also decreased oxidative stress in cultured cells [25], but cytotoxicity was not evaluated. Homocysteine, when incubated with aorta endothelial cells, produced oxidative cell injury and lipid peroxidation, a result of mitochondrial toxicity (decreased respiration and RNA levels) induced by synergism between homocysteine and H$_2$O$_2$ generated by autoxidation [26]. In hypertensive patients short-term supplementation with folic acid decreased oxidative stress biomarkers [27].
Folate can prevent mitochondrial toxicity induced by methanol metabolites (Fig. 1). Methanol is oxidized to formaldehyde catalyzed by alcohol dehydrogenase ADH1 which is then oxidized to formate catalyzed by mitochondrial aldehyde dehydrogenase ALDH2 or cytosolic GSH dependent formaldehyde dehydrogenase (ADH3). Formaldehyde and formate are inhibitors of mitochondrial respiration [28]. Although research is required to substantiate this, formate formed in the cytosol and mitochondria is presumably detoxified by its respective 10-formyltetrahydrofolate synthetase and 10-formyltetrahydrofolate dehydrogenase activities (enzymes 5 and 7, Fig. 1). Folate deficient patients might thus be expected to be more susceptible to methanol or formalin. In fact, whilst the current antidote used in poison control centers for methanol poisoning is ethanol or an ADH inhibitor, folic acid (leucovorin) therapy has recently been added to the American Academy of Clinical Toxicology practice guidelines on the treatment of methanol poisoning [29]. Folate may also be useful against formaldehyde toxicity as its major metabolite, tetrahydrofolate, forms the condensation product 5,10-methylenetetrahydrofolate (CH$_2$THF) with formaldehyde [30].

Folate may affect riboflavin status. After folic acid supplementation, riboflavin status was decreased in volunteers, while after a folate-enriched diet, riboflavin levels were improved [31]. This was suggested as showing that high folate intakes may increase the FAD requirement for optimal activity of MTHFR, thus decreasing the circulating levels of FAD.

Anti-folates have been important in cancer therapy and folate has been considered one of the more promising chemopreventive agents. Individuals with the highest folate intake have a 30–40% lower risk of developing colorectal cancer than those with a lower intake of folate [32]. Folate deficiency increases colon carcinogenesis in mice and rats in some model systems [33] but decreases colon carcinogenesis in others (e.g. [34]). However in recent clinical trials supplementary folic acid increased the frequency of recurrent colonic polyps [35]. This ambiguity in the relationship between folate deficiency and the risk of colon carcinogenesis both in epidemiological studies and animal models was recently reviewed [32]. Folate is essential for the synthesis, repair and methylation of DNA and folate deficiencies increase their induction of mutations and result in the induction of early steps of carcinogenesis. The vitamin requirement increases at the site of the tumor due to the higher rate of cellular divisions, and while folate intake may prevent the early development of cancers, it may also increase the growth of the tumor once established. The requirement for folate coenzymes for purine and thymidylate synthesis has led to the use of antifolate drugs e.g. methotrexate (inhibitor of dihydrofolate reductase (reaction 1, Fig. 1) and mitochondrial folate uptake) or fluorouracil (inhibitor of thymidylate synthase (reaction E, Fig. 1)) in the treatment of cancer. These agents can inhibit tumor cell proliferation and induce mitochondrial toxicity and apoptosis [36]. Methotrexate is still a drug of choice for treatment of childhood leukemia in combination with citrovorum factor (leucovorin). This combinational therapy allows very high doses of methotrexate to be used for optimal anticarcinogenic effect, followed by the antidote leucovorin to prevent side effects and apoptosis of normal tissues. After more than 40 years of use, the controversy over this use of combination methotrexate therapy is still strong [37].

2.5. Choline as an alternate methyl group donor

Dietary choline was first shown to be important more than 70 years ago. Choline prevented the development of fatty livers in rats fed diets with fat devoid of choline. Later it was shown that a deficiency of dietary choline in animals resulted in liver cancer, and that such diets also promote breast, colon and other cancers [38]. Choline was also found to be necessary for the growth of mammalian cells in vitro.

As shown in Fig. 2, choline is oxidized in the mitochondria to betaine aldehyde, catalyzed by an unidentified inner membrane oxidase (choline dehydrogenase (reaction 1, Fig. 2)), and then to betaine (N,N,N-trimethylglycine) catalyzed by NAD$^+$ and betaine aldehyde dehydrogenase (reaction 2, Fig. 2). Betaine is a major mitochondrial methylating agent which maintains cellular replication, liver function and detoxifies some phenolic xenobiotics. Betaine can also be used to decrease plasma homocysteine levels as betaine instead of methyltetrahydrofolate can be used to re-synthesize methionine from homocysteine, catalyzed by betaine: homocysteine methyl transferase (reaction 3, Fig. 2) [39]. Choline metabolism by sequential demethylation (reactions 4–7, Fig. 2) allows an alternative pathway for CH$_2$THF regeneration from THF in cases where MTHFR activity is low.

Choline can provide methyl groups for DNA synthesis. As the dietary requirement for choline is reduced by increased folate, methionine and vitamin B12, it has become customary to consider all these dietary components together as sources of methyl donors. There appears, however, to be a specific requirement for dietary choline, independent of the other methyl donors [40]. Current research on methyl group deficiency is largely
focused on the effects of a deficiency of methyl donors on DNA methylation processes and on mutations resulting from misincorporation of uracil in DNA. There are dramatic reductions in genome-wide methylation occurring with proliferation [41] and gene-specific methylation has been associated with carcinogenesis [42]. Mutational events also occur, though they appear to primarily affect mitochondrial DNA [39]. These effects, however, may not explain liver carcinogenesis with choline-specific deficiency.

Recent studies suggest that liver failure of choline deficiency (steatohepatitis) is a consequence of reduced phosphatidyl choline and reduced membrane integrity [43]. Choline deficiency may increase oxidative stress in liver cells. It increases lipid oxidation in the nuclear fraction of liver cells [44] and 8-hydroxyguanine in DNA [45], though it did not increase the malonyldialdehyde adduct [46]. Colon cancer can be induced in mice a diet with reduced choline, folate and methionine, and a similar diet with reduced calcium and vitamin D3 [47] but this diet did not induce mutations in colonic cell DNA [48]. The mechanism of carcinogenesis thus appears to be quite complex.

2.6. The central role of serine and glycine

Unlike other amino acids that are oxidized by the TCA cycle, glycine is poorly oxidized by mammalian mitochondria and is thus a poor energetic substrate. In the mitochondria glycine readily methylates tetrahydrofolate with the aid of the mitochondrial glycine cleavage enzyme system to form CH₂THF, CO₂ and NH₃, which is detoxified by the urea cycle. Glycine is also converted to serine for purine and phospholipids synthesis.

But glycine also plays roles unrelated to its carbon-transfer potential: (1) both serine and glycine are required as building blocks for the de-novo synthesis of glutathione (GSH), an essential cellular antioxidant molecule (Fig. 3); (2) glycine is used in the cytosol for protein, sarcosine and creatine synthesis. Creatine and its high-energy creatine phosphates act as a muscle energy store; (3) glycine provides nitrogen for the synthesis of other amino acids; (4) glycine is a building block in the formation of aminolevulinate, an intermediate of heme synthesis; (5) finally glycine forms glycine conjugates (hippuric acids) with carboxylic acids catalyzed by N-acetyl transferase.

Glycine availability in the cell is highly dependent upon the one-carbon pathways. The reverse statement is also true. Folate and choline metabolism is dependent upon supplies of glycine/serine. Glycine may therefore play a role in a large number of essential pathways in the mitochondria and the cell. In its other functions, glycine is central to the antioxidant defenses of the cell as well as mitochondrial respiration and energy production pathways. Therefore glycine or serine deficiency, due to
either folate or choline deficiency can lead to increased risk of oxidative stress to the cell.

### 3. Cobalamin (vitamin B12)

Cobalamin was isolated and identified in the late 1940s. Its potential as an antiperinicious anemia factor, however, had been recognized 20 years previously, with the treatment of patients with fresh liver meat and liver extracts. Other conditions associated with cobalamin deficiency are listed in Table 2. Only bacteria or microorganisms synthesize cobalamin and animals and plants do not. The human need is very small, i.e. about 1 μg/day. B12 deficiency has been shown to affect 10–15% of humans over the age of 60 in the US largely due to abnormalities in B12 uptake from the gut, resulting in a functional B12 deficiency [49].

#### 3.1. Cobalamin delivery from the diet to mitochondrial enzymes

Cobalamin is present in organ meats, milk and milk products, shellfish and other fish. There are three forms in our diet. Adenosylcobalamin (coenzyme B12), methylcobalamin, and hydroxycobalamin are found in fish and meat and particularly liver, whereas milk and cheeses contains mostly hydroxycobalamin. Strict vegetarians need to take cobalamin regularly as a supplement.

Cobalamins are released from dietary proteins by the acid environment and pepsin in the stomach. In both cases, the gastric and early small intestine pH can be increased to levels that prevent dissociation or absorption of cobalamin. The risk of cobalamin deficiency, even with a cobalamin-rich diet, can thus be increased in the ageing population [50] and for chronic users of antacid drugs [51]. Once released from dietary proteins, cobalamins bind to salivary R binder polypeptide. The binder protein is digested in the small intestine by pancreatic trypsin and the cobalamins are transferred to stomach-derived intrinsic factor (IF) forming a IF/Cbl complex. This complex interacts on ileal mucosa by endocytosis to a lysosomal vesicle [52]. In the lysosomes, the complex is dissociated by an unknown mechanism and cobalamin, likely as hydroxycobalamin, crosses the basal membrane bound to newly synthesized transcobalamin II into the portal blood [52]. In the hepatocyte, TC/Cbl complex is recognized by transcobalamin receptors and leads to endocytosis into the lysosomes and endosomes where proteolytic digestion release hydroxycobalamin. Hydroxycobalamin is released in the cytosol and converted to the active coenzyme methylcobalamin via methionine synthase reductase, as part of the methionine synthase complex. It is not known how hydroxycobalamin enters the mitochondria. In the mitochondria the cobaltic complex of hydroxycobalamin is reduced by reductase/GSH and is then converted by adenosyl transferase and ATP to form cobaltous adenosylcobalamin that can bind as a coenzyme to methylmalonyl-CoA mutase [53]. Cobalamins are released by the hepatocyte into the plasma as methylcobalamin bound to transcobalamin I or haptocorrin. This prevents loss by filtration through the kidneys but allows tissues to take up cobalamin via asialoglycoprotein receptors. While haptocorrin-bound cobalamin represents 80–90% of circulating form of vitamin B12, its role is unclear [54]. Cellular uptake of cobalamin is mostly dependent on presentation with transcobalamin.

#### 3.2. Essential role of B12 in mitochondrial energy production and cellular function

Cobalamin is involved in one-carbon transfer pathways as shown in Fig. 1. Methylcobalamin and CH3THF act as cytosolic methyl carriers for methionine synthase which catalyses the conversion of homocysteine to methionine (reaction 9, Fig. 1). Tetrahydrofolate produced in this reaction can receive a one-carbon unit as CH2 from serine. The resulting CH2THF is required to synthesize deoxythymidine monophosphate (dTMP), a DNA precursor. The exact cause of megaloblastic anemia associated with cobalamin deficiency is not known but it is likely a consequence of the impairment of DNA synthesis rather than hemoglobin synthesis. It has also been suggested that the methionine synthetase inhibition in B12 deficiency causes an accumulation of methyltetrahydrofolate that, in turn, results in a functional folate deficiency and an inhibition of thymidylate synthetase.

In the mitochondria adenosylcobalamin is required for the synthesis of succinyl-CoA from the carbon atoms of valine, isoleucine, methionine or metabolites of odd-numbered fatty acids. The propionyl-CoA formed is converted to \( l \)-methylmalonyl-CoA which undergoes an adenosylcobalamin dependent isomerization to succinyl-CoA, an important intermediate of the TCA cycle. Adenosylcobalamin is formed in the mitochondrial matrix from reduced hydroxycobalamin and ATP catalyzed by adenosyl transferase.

#### 3.3. Cobalamin deficiency

Vitamin B12 status is commonly assessed by mean corpuscular volume or serum or plasma vitamin B12. Its
status can be inferred from concentrations of methylmalonic acid (MMA), homocysteine, formiminoglutamic acid, propionate, methylcitrate levels or holotranscobalamin II.

Dietary B12 deficiency is difficult to induce in animals. It requires long periods on special diets and yields a heterogeneous metabolic defect. Although rats on a cobalamin deficient diet for 24 weeks do not develop megaloblastic anemia they do develop methylmalonic aciduria and neuropathy [55]. Cobalamin deficient rats will, however, develop megaloblastic anemia-like symptoms if they are exposed to 10% oxygen for an additional 6 weeks so as to induce increased hematopoiesis [55]. Cobalamin deficiency also affects folate metabolism. Rats on a cobalamin deficient diet have a methionine synthetase inhibition. The resulting hypomethylation could explain the anomalies in the base substitution and decreased methylation of DNA [56] and could explain the increased liver and colon carcinogenesis observed with B12 deficiency [57,58]. Cobalamin deficiency also leads to increased CH3THF and decreased CH2THF levels, resulting in increased dUMP production and misincorporation into DNA. Cobalamin deficiency inhibits methionine synthase, thus leading to increased homocysteine concentrations [19]. Cobalamin deficiency does not affect free folate levels in patients but is associated with decreased levels of polyglutamate folate, suggesting inhibition of synthesis of the folate coenzyme form. The consequence is an inhibition of single unit carbon transfer required for DNA and RNA synthesis, glycine to serine conversion, histidine breakdown and homocysteine to methionine conversion [59].

Cobalamin-dependent metabolism has multiple metabolic effects although only two enzymes have an absolute cobalamin coenzyme requirement. Inhibition of methylmalonyl-CoA mutase (deoxyadenosylcobalamin dependent enzyme) leads to increase of plasma MMA and increased urinary excretion [60]. The accumulation of serum MMA is a reliable test of cobalamin deficiency or hereditary methylmalonyl aciduria. Methylmalonic acidemia and mitochondrial toxicity due to MMA excess lead to inhibition of carbamyl phosphate synthetase I, pyruvate carboxylase and the dicarboxylate carrier required for the malate shuttle (reviewed in [61]). Defect in N-acetylglutamate synthase, glycine cleavage enzyme and pyruvate carboxylase can result in hyperammonemia, hyperglycinemia and hypoglycemia, respectively. There can also be increased hepatic pyruvate oxidative metabolism and lactic acidosis [62]. Methionine synthase is dependent on both CH3THF and cobalamin. Inhibition of this enzyme leads to increased levels of homocysteine and metabolites such as cystathionine [15], as well as decreased folate metabolism [63]. In the rat, cobalamin deficiency decreased liver activity of L-serine dehydratase [63] with accumulation of the upstream choline metabolite, dimethylglycine [15]. B12 deficiency also increased plasma levels of serine, threonine, glycine, tyrosine, lysine, histidine [63], methylcitric acid and propionyl-CoA [62] as well as increased NAD+ to NADH ratio in the liver.

MMA accumulation is also hypothesized to affect mitochondrial respiration. MMA was reported to inhibit complex II and β-hydroxybutyrate dehydrogenase [64,65]. After only 2 weeks of cobalamin deficiency (induced by cobalamin analogue), liver mitochondrial mRNA expression was reduced. Polycistrionic mRNAs accumulated and by 5–6 weeks the activity of complex III (cytochrome b) and IV (cytochrome a/a3) of the electron transport chain was decreased [66]. Cobalamin deficiency increased mitochondrial matrix enzymes such as citrate synthase and succinate dehydrogenase. It also inhibited the electron transport chain and state-3 oxidation via glutamate, succinate, or duroquinol, and increased CoA and fatty acid synthesis [67]. The findings linking cobalamin deficiency and mitochondrial respiration are ambiguous, the authors suggesting that complex II inhibition may be a consequence of the accumulation of MMA metabolites methylocitric acid (MCA) and malonic acid (MA) rather than a response to MMA itself [68]. The exact nature of the inhibitor however does not contradict earlier findings as all three metabolites are increased in methylmalonic aciduria [64]. More investigations are still needed to fully understand the physiological impact of cobalamin deficiency on the electron transport chain and mitochondrial energy pathways.

3.4. Prevention of oxidative stress and mitochondrial toxicity by cobalamin

There have been no reports that vitamin B12 prevents mitochondrial toxicity or has antioxidant properties, though B12 is known to be an efficient radical trap [69]. Cobalamin deficiency however has been shown to cause mitochondrial toxicity as a result of inhibition of the TCA cycle [70]. In megaloblastic anemia red blood cell hemolysis is frequently high. There is frequently hyperhomocysteinemia, lipid peroxidation and evidence of protein bound homocysteine in red blood cell ghosts, suggesting that cobalamin deficiency can result in oxidative stress-induced cell death from high intracellular concentrations of homocysteine [71]. Vitamin B12 can enhance the effectiveness of folate to reduce high blood homocysteine levels and oxidative stress. Indeed cobalamin supplementation of patients with end stage renal
disease can decrease plasma homocysteine, MMA excretion and serum folate levels without having an effect on erythrocyte folate [72]. Currently there are more than 20 prospective, worldwide, interventional trials involving at least 100,000 participants that are examining the effect of supplementary B-vitamins on the concentration of plasma homocysteine and morbidity and mortality from atherosclerotic vascular disease [73].

4. Pyriodoxal (vitamin B6)

The term “vitamin B6” covers the three vitaminers: pyridoxal, pyridoxine and pyridoxamine (Table 1). Pyridoxine, pyriodoxal (PL) and pyridoxal phosphate (PLP) are the major dietary forms of the vitamin. Pyridoxine was first identified in 1938. The catalytically active aldehyde (pyriodoxal) or amine (pyridoxamine) and their phosphates were discovered in the early 1940s. Vitamin dependency was first recognized in 1954 with a recessively inherited condition in which children develop epileptic seizures beginning early in life [74]. Clinical evidence of vitamin B6 deficiency is not common because of its widespread occurrence in foods but Table 2 gives conditions associated with deficiency of the vitamin. Approximately 10% of the US population consumes less than half of the RDA for B6 and could be at an increased risk of cancer, neural decay and accelerated aging [49].

4.1. Vitamin B6 delivery from the diet to mitochondrial enzymes

Vitamin B6 is synthesized by plants and products that are particularly rich in B6 are vegetables, whole grain cereals, nuts, and muscle meats. An increased the risk of B6 deficiency can be observed in advanced age, severe malnutrition, excessive alcohol ingestion, and in dialysis. Thermal losses of B6 in food processing and preservation vary from 49% to 77% in canned foods or infant formula probably because any protein lysine present reacts with pyriodoxal at 37°C to form a Schiff base which rearranges to ε-pyriodoxylidenelysine as shown in Fig. 4 [75,76].

Drugs such as isoniazid, cycloserine, hydralazine and penicillamine, or the anti-Parkinson drug levo-dopa can also deplete tissue B6 levels. B6 deficiency can be assessed with the in vivo rat tryptophan loading test, which shows a marked increase tryptophan conversion to xanthurenic acid which is reversed by the concomitant administration of pyridoxine. Pyriodoxal kinase inhibition is also involved, and although the mechanisms involved are not known and poorly understood, it is believed that this B6 antagonist effect may be because the drug reacts with PLP to form a Schiff base, which depletes cytosolic B6 levels (and inactivates the drug). Furthermore the Schiff base hydrazone products formed with hydrazine carbonyl reagents or drugs (e.g. isoniazid, semicarbazide, hydrazine and hydroxylamine) compete with B6 for the B6 enzyme binding site and prevents the formation of the PLP coenzyme [77]. Other mechanisms of pyriodoxal kinase inhibition by drugs have been described [78]. The in vivo formation of a Schiff base of the antidiabetic drug aminoguanidine with pyriodoxal as shown in Fig. 4 has also been described [79]. The increase in tryptophan metabolism to xanthurenic acid could be because B6 dependent enzymes involved in the competing tryptophan metabolic pathway to anthranilic acid and nicotinic acid were inhibited. Furthermore the peripheral neuropathy induced in some patients by the chronic administration of isoniazid for tuberculosis can be prevented by pyridoxine administration. Similar results were obtained for phenelzine or hydralazine. Indeed excessive supplementation with B6 can affect the efficacy of these drugs, e.g. levo-dopa, hydralazine, phenytoin [77].

Vitamin B6 is absorbed from the diet mainly by simple diffusion across the enterocyte brush-border of the jejunum. Enterocyte and other cell plasma membranes are not permeable to PLP, and PLP needs to be dephosphorylated by intestinal phosphatases prior to being taken up by enterocytes [80]. The three B6 vitamers are then phosphorylated (i.e. metabolically trapped) in the enterocyte but require dephosphorylation
by plasma membrane phosphatases (similar to alkaline phosphatases) before being released into the portal vein for transport to the liver. About 10% of the total body pool of PLP is in the liver and about 20% of cellular B6 is located in the liver mitochondrial matrix as PLP. Pyridoxine, pyridoxal and pyridoxamine are taken up rapidly by hepatocytes and as shown in Fig. 5 is accumulated by phosphorylation metabolic trapping catalysed by the kinase (reaction 1, Fig. 5). Kinases first form pyridoxine-5′-phosphate and pyridoxamine-5′-phosphate. These are oxidised at the 4′ position by a cytosolic pyridoxine phosphate oxidase (reaction 2, Fig. 5) that requires flavin mononucleotide (FMN) and O2. The PLP formed is then transferred to PLP-dependent apoenzymes [81,82].

Fig. 5. Metabolism of B6 vitamins: metabolic conversion of B6 vitamins catalyzed by: (1) pyridoxal (PL) kinase, (2) pyridoxal phosphate (PLP) oxidase, (3) B6 vitamin kinase conversion can be reversed by phosphatases, (4) any unbound pyridoxal is oxidised by aldehyde oxidase/aldoxyde dehydrogenase 2 (ALDH2) to form pyridoxic acid which is released into the plasma and excreted in the urine.
The kinase is more active with PL than pyridoxine in humans and more active with PL than pyridoxamine in rats [82]. Pyridoxamine incubated with hepatocytes undergoes similar steps to form PLP with pyridoxamine phosphate oxidase activity [82].

Recently the 3D structure of pyridoxal kinase from sheep brain and its complex with ATP has revealed that it is a dimer and that pyridoxal binding causes the protein to become more compact and the pyridoxal more tightly bound [83]. The kinase also forms complex with the oxidase and transaminase that prevents the release of free PLP and hydrolysis by phosphatases. The 3D structure of the oxidase shows it is also a dimer containing an essential arginine, required for binding of PLP. The oxidase is inhibited by dicarbonyls. Mitochondria have no kinase or pyridoxine/pyridoxamine-P oxidase activities so pyridoxal phosphate is taken up by liver mitochondria by passive diffusion facilitated by protein binding which acts as a store [84]. Mitochondria may also take up pyridoxamine-P by passive diffusion as mitochondria contain similar amounts (45% each) of pyridoxal-P and pyridoxamine-P whereas pyridoxine-P only accounts for 2% of the B6 vitaminers [85]. [14C]pyridoxine incubated with hepatocytes forms mostly PLP, and releases both PL and PLP into the plasma [86]. Presumably PLP is released as an albumin complex to the plasma. PLP also binds avidly to other plasma proteins such as transferrin, or α-glycoproteins through formation of Schiff bases (aldimine). Alternatively, the hepatocyte can release PLP as the albumin complex.

Any unbound pyridoxine or pyridoxal in the hepatocyte is oxidatively inactivated by aldehyde oxidase/aldehyde dehydrogenase 2 (ALDH2) to form 4-pyridoxic acid which is released into the plasma and is excreted in the urine [82]. Pyridoxic acid is mostly the only B6 vitamer found in the urine. The rate constants for the various steps in Fig. 4 and proposed for rat and human liver show that the kinases/oxidases (reactions 1 and 2, Fig. 5) are faster than the phosphatases (reaction 3, Fig. 5) [82].

Plasma levels of B6 vitamers in order of concentration are PLP > pyridoxine > pyridoxic acid >> pyridoxal with much lower levels for pyridoxine-P, pyridoxamine-P or pyridoxamine [86]. By contrast pyridoxamine-P and PLP are the major B6 forms found in the tissues and the mitochondria. The intracellular pathways of pyridoxamine have been much less studied and it has not yet been demonstrated that mitochondria take up pyridoxamine-P. Presumably the pyridoxamine is not released from liver cells as plasma pyridoxamine or pyridoxamine-P levels are much lower than the other B6 vitaminers. How PLP is released by hepatocytes remains controversial. Finally it is not known which of the B6 vitaminers added to cells is the best at preventing cytotoxicity induced by mitochondrial toxins.

4.2. Essential role of vitamin B6 in mitochondrial and cellular function

Vitamin B6 is involved in aminotransferase reactions. Mitochondrial function is more dependent on PLP than other subcellular organelles for a number of reasons. PLP functions as a coenzyme for transaminases that participate in the catabolism of all amino acids by the urea cycle of the mitochondria. Accordingly the human requirement for pyridoxine increases as protein intake increases. The chemical basis for PLP catalysis is to enable its carbonyl group to first form a Schiff base with the amine component of the amino acid substrate. The protonated form of the pyridoxal phosphate then acts as an electrophilic catalyst, before the Schiff base is cleaved. Transaminases are of two types in the way they utilize PLP: either forming α-oxo acids from α-amino acids, or forming primary amines via its decarboxylase catalytic action. Not all transaminases, however, are inhibited during pyridoxine deficiency. Transaminases also participate in the malate aspartate shuttle that enables mitochondria to oxidize NADH formed by glycolysis. Transaminases can also link amino acid metabolism to energy production through two reactions: first alanine can form pyruvate, feeding into the glycolysis pathway, and second glutamate can form α-ketoglutarate, feeding directly into the TCA cycle.

Vitamin B6 is also involved in decarboxylation reactions. Pyridoxal phosphate is a coenzyme for aminolevulinate synthase located in the mitochondrial matrix, which catalyses the initial and rate limiting step for heme synthesis from glycine and succinyl-CoA. The synthase requires rapid synthesis as it has a short half-life (1 h) probably because the aminolevulinate product can inactivate the synthase by reacting with the pyridoxal carbonyl or can form ROS by autoxidation. Thus a B6 deficiency could increase cellular endogenous ROS formation because of a heme/cytochrome deficiency. For instance, a deficiency of mitochondrial complex IV could impair respiration and accelerate the mitochondrial or cell aging process [49,87].

Vitamin B6 is a cofactor for enzymes involved in side-chain cleavage reactions and as such can play a role in a large number of pathways: (1) PLP is a cofactor for two enzymes involved in cysteine formation, as detailed in Fig. 3: the hemoprotein cystathionine β-synthase (CBS (reaction 1, Fig. 3)) that removes serine from homocysteine to form cystathionine, and γ-cystathionase (GCT...
(reaction 2, Fig. 3), that then forms cysteine. Vitamin B6 is coenzyme of γ-cystathionase. It releases α-ketoglutarate that can feed into the tricarboxylic acid (TCA) cycle, increasing production of NADH later used for energy metabolism [2]. In this respect the action of vitamin B6 is similar to that of vitamin B12, which is a coenzyme of methylmalonyl-CoA mutase that leads to the formation of succinyl-CoA that also feeds into the TCA cycle as noted above. Vitamin B6 deficiency does not decrease the activity of CBS but does decrease the activity of GCT; (2) cysteine sulfinate decarboxylase, which catalyzes taurine formation, also loses its activity rapidly during pyridoxine deficiency; (3) serine hydroxymethyl transferase (reaction 2, Fig. 1) is another PLP-dependent enzyme. It catalyzes the three-carbon transfer from serine to tetrahydrofolate to form 5,10-methylenetetrahydrofolate (Fig. 1), an important step to help remethylate homocysteine; (4) PLP is also required for the metabolism of tryptophan to NAD⁺ as it is a coenzyme for kynureninase and kynurenine aminotransferase that catalyze intermediate reactions [2]; (5) glycine formation from glyoxylate is also catalyzed by a PLP-dependent transaminase; (6) vitamin B6 is a coenzyme of aminolevulinate synthase and thus B6 deficiency can prevent heme formation and cytochrome c synthesis, inhibiting the mitochondrial respiration at complex III of the electron transfer chain (reviewed in this issue [2]).

The aldehyde group of PLP is much more reactive than the aldehyde group of pyridoxal which is mostly hydrated. Pyridoxal phosphate binds extensively to proteins and enzymes by forming a Schiff base with lysine ε-amino groups (Fig. 4) which can inhibit their activity in vitro e.g. succinic semialdehyde reductase, aldehyde reductase, aldose reductase, acetyl-CoA carboxylase activity [88]. Whether this occurs under physiological conditions or contributes to pyridoxal induced peripheral sensory neuropathy toxicity is unknown. The depletion of perfused liver or hepatocyte pyridoxal phosphate levels by ethanol has been attributed to the acetaldehyde metabolite displacing protein lysine bound pyridoxal phosphate [89]. Most (80%) PLP in the body is in the muscle where it is stored attached to the phosphorylase enzyme, and is released during food deprivation, but not marginal pyridoxine deficiency. PLP binds to albumin for storage, thus conditions associated with low albumin levels are also likely to show low pyridoxal activity [90].

4.3. Vitamin B6 deficiency

Vitamin B6 status is commonly measured by determining plasma, erythrocyte or total blood PLP, or by determining urinary pyridoxal excretion. It can be assessed by erythrocyte tryptophan catabolites levels, aspartate aminotransferase and alanine aminotransferase activities, or plasma homocysteine. Pyridoxine deficiency can also be diagnosed by increased urinary 4-oxalate, after a load test of 2 g oral tryptophan. Urinary cystathionine and plasma homocysteine levels are also measured after a test dose of 3 g methionine [3].

By-products of pyridoxal-dependent reactions could provide a further test of vitamin status. A deficiency of pyridoxine does not induce a significant increase in homocysteine under normal conditions [91], though it does increase homocysteine, cystathionine and glycine to abnormal levels after a methionine load. This suggests that under normal conditions these pyridoxal dependent pathways are not limiting [15]. A deficiency of pyridoxine could also inhibit aromatic-β-amino acid (dopa) decarboxylase and thereby decrease the concentration of dopamine in the brain [92].

Pyridoxine appears to be involved in inflammation. In rheumatoid arthritis, vitamin B6 is hypothesized to be mobilized from the liver and peripheral tissue to the site of inflammation [90]. There also appears to be an inverse correlation between PLP and C-reactive protein in the plasma, independent of homocysteine levels [93], which has suggested that PLP is used as a coenzyme in inflammation reactions [94] or that B6 deficiency results in inflammation. Smokers and patients with chronic inflammation or advanced age have high levels of interleukin 6 (IL-6) that inhibit pyridoxal phosphatase. The decrease in liver PLP would inhibit cystathione β-synthase and moderately increase homocysteine in the liver and plasma [95].

Oxalate metabolism is also affected by vitamin B6. A vitamin B6 deficient diet induced lipid peroxidation in the liver and increased liver lipids, oxalate, calcium and iron levels in rats. This also depleted liver ascorbate, tocopherol and GSH levels and decreased catalase activity [96]. The increased oxalate levels were attributed to decreased PLP-dependent alanine: glyoxalate transaminase which increased glyoxalate levels and thus glycolate oxidase activity. Excessive oxalate cannot be metabolized and is eliminated by renal excretion. High urine oxalate resulted in calcium oxalate accumulation in the kidney, and mitochondrial toxicity [97], loss of the renal function and significant morbidity. Calcium oxalate in the mitochondria can open the mitochondrial proximal tubules, thus leading to swelling of the organelle and inhibition of the mitochondrial electron transport chain (likely at stage 3) [97]. Pyridoxine benefits 50% of patients with primary hyperoxaluria type I, involving the mistargeting of PLP-dependent alanine:...
glyoxalate transaminase I to mitochondria instead of the peroxisomes [98].

4.4. Prevention of oxidative stress and mitochondrial toxicity by vitamin B6

Vitamin B6 can have a direct antioxidant activity as pyridoxamine was more effective than pyridoxine at preventing superoxide radical formation, glycated hemoglobin formation and erythrocyte lipid peroxidation during glucose autoxidation [99]. Pyridoxamine was also more effective than pyridoxal at preventing H₂O₂ induced mitochondrial toxicity in monocytes whereas pyridoxal was more effective than pyridoxamine at inhibiting superoxide radical formation [100]. Vitamin B6 can also have indirect effects through chelating activity. Pyridoxine prevented chromium-induced kidney oxidative stress and toxicity in rat [101]. Pyridoxal also induced iron excretion in the rat largely because of its iron chelating activity [102] and pyridoxamine prevented copper induced hepatocyte oxidative stress cytotoxicity [103]. Of particular interest, pyridoxine alone (100 mg/kg oral administration) increased rat kidney GSH levels by 57% and ascorbate levels by 64% after 12 h [101]. Supplementary pyridoxine decreases the formation of colon tumors in mice given repeated injections of azoxymethane [104]. The mechanism is not known, nor is it known whether the antioxidant properties of B6 result from the reaction of peroxyl radicals with the hydroxyl and/or the amine groups on the pyridine ring.

Vitamin B6 may have antiabetic antioxidant properties. Pyridoxine therapy was first described when vitamin supplementation was used to delay peripheral neuropathy in diabetic patients, presumed to be exposed to increased oxidative stress [105]. Pyridoxamine also prevented the development of retinopathy and nephropathy in streptozotocin-induced diabetic rats [106,107]. Indeed pyridoxamine is more effective than the antidiabetic agent aminoguanidine at preventing the nonenzymatic oxidative glycation of proteins by glucose. This antioxidant property has been attributed to the amine group of pyridoxamine that forms a Schiff base linkage with the carbonyls of open-chain sugars, dicarbonyl fragments (e.g. glyoxal and methylglyoxal), Amadori products and post-Amadori intermediates [108,109]. Pyridoxamine thus not only acts as a scavenger of toxic carbonyl products of glucose and lipid peroxide degradation products which would otherwise covalently bind to proteins, but also prevents the formation of advanced lipoxidation endproducts (ALE) or glycation endproducts (AGE) by blocking oxidative degradation of the Amadori intermediate of the Maillard reaction. This activity suggests that pyridoxamine derivatives could act as AGE breakers in vivo though AGE products are usually thought to remain irreversibly bound to proteins. Collagen cross-linking increases with age and is accelerated with diabetes. Pyridoxamine also traps the α-dicarbonyl intermediates formed during lipid peroxidation that bind to proteins to form ALE without inhibiting fatty acid peroxidation. Pyridoxamine adducts are excreted in the urine [110,111]. ALE are increased in diabetes and hyperlipidemia and likely contribute to the development of long-term renal and vascular pathology. Currently pyridoxamine is on the FDA “fast track” to phase III clinical trials for treatment of diabetic nephropathy [112].

Ethylene glycol-induced renal mitochondrial toxicity, as described by inhibition of mitochondrial respiration and loss of mitochondrial structure, has been attributed to its glyoxalate metabolites that can form oxalomalate, an aconitase inhibitor, and oxalic acid, an activator of the permeability transition pore [97]. The responsiveness of renal failure to pyridoxine can be attributed to increased intracellular PLP binding to the transaminase which catalyses glyoxalate conversion to glycine thereby preventing the accumulation of glyoxalate and oxalic acid. Pyridoxine (5 g) is therefore recommended as an antidote for fatal kidney poisoning by ethylene glycol [113].

There may be safety risks associated with chronic excessive pyridoxine administration. Multivitamin supplements typically provide about 4 mg of B6 per day. Symptoms of peripheral sensory neuropathy attributed to B6 toxicity have been observed with chronic dosing at or above 50 mg/day for 6–60 months with full recovery after cessation of the treatment [114]. Peripheral neuropathy was also induced in dogs following 50–200 mg/kg daily for 40–75 days.

5. Conclusions and future perspectives

The transfer of methyl groups to target biomolecules is a complex process. The molecular carriers are metabolites of folate and choline, but the formation and activation of these metabolites depends on cobalamin and vitamin B6 as well as other vitamins. As a result, the cellular pathways for methyl transfers can be disrupted by vitamin deficiencies in more than one way, and detrimental effects resulting from the disruptions can be similar for the different vitamin deficiencies. There is less overlap of pathways for choline than for folate. The details of the vitamin B6 and cobalamin pathways and the specificities of choline-dependent reactions mean that choline alone cannot be considered a sufficient supplementation option for a deficiency of folate, B6, or B12. Furthermore, tar-
geted utilization of choline resources for methyl transfer could deflect from other choline-dependent pathways.

This review of the scientific literature shows that B vitamins play an essential role in mitochondrial functions and suggests that mitochondrial functions are compromised by dietary B vitamin deficiency or increased B vitamin dependency. However the literature is limited and in many cases based on methods which have since been substantially improved. Further research using more refined methods is now needed to define the effects of vitamin B deficiencies on mitochondrial function over a range of environmental and genetic conditions. Some examples of possible approaches are:

(1) New techniques such as proteomics and metabolomics could add greatly to our understanding of vitamin deficiencies. Vitamin deficiencies can result in the accumulation of potentially reactive intermediates which can form adducts on circulating proteins and target tissues. We might ask, for example: Do B vitamin concentrations affect the levels of sulfolactone adduct concentrations in plasma? Are there significant differences in the concentration of these homocysteine adducts in different tissues? Are other adducts formed with other vitamin deficiencies?

(2) In vitro mammalian cell screening methods could be used to evaluate interactions between B vitamins and xenobiotics. This might include evaluation of protections against cell toxicity induced by xenobiotic toxins, identification of interactions reducing the levels of B vitamins, and identification of target subcellular organelles or target different metabolic pathways that cause different molecular cytotoxic mechanisms. For example: Vitamin B6 is reduced by interactions with penicillamine. Are there other conditions in which B vitamin activity is lost and is this loss ever tissue specific?

(3) In vitro cellular and in vivo animal studies could clarify the degree to which cellular pathways are compromised by deficiencies and the most effective combinations of these factors in reducing possible risk. For example: Is there an optimal combination of folate, vitamin B6 and cobalamin to reduce the accumulation of homocysteine? What are the optimal cellular and tissue concentrations of vitamins to assure optimal enzyme activity?

(4) Spontaneous genetic mutations of specific enzymes (polymorphism) could be further investigated to assess the degree to which daily vitamin requirements of individuals require a targeted approach. There is thus a need to identify further specific vitamin biomarkers to identify individual vitamin deficiencies both to provide diagnostic biomarkers for examining general pathways and products, and as molecular or genetic tools to understand the consequences and mechanisms of marginal vitamin supply in population studies. For example: Could studies of polymorphisms provide an aid in the identification of B vitamin requirements?

(5) Genetic approaches with knock-out murine models could be used to better understand specific vitamin-dependent reactions and pathways. Knock-out murine models have been developed for folate, vitamins B12 and B6 at the level of absorption, metabolism and vitamin-dependent reactions. As folates are substrates for one-carbon transfer reactions, not for coenzymes, research has thus focused on absorption and metabolism. Presently there are knock-out models of Folate binding protein (Folbp1) and Reduced folate carrier (RFC1) [115] as well as of methylene tetrahydrofolate reductase (Mthfr), a central folate metabolic enzyme [116]. Cobalamins have also been investigated both for absorption, via cubulin receptors [117], and the two cobalamin-dependent mammalian enzymes; methionine synthase (MS) [118] and methylmalonyl-CoA mutase (mut) [119]. Vitamin B6 investigations, however, have been limited to an alkaline phosphatase (TNAP) knock-out [120]. There are a number of further genes that could be targeted. Folate is involved in a very large network of reactions and enzymes such as serine-hydroxymethyl transferase (transport of the C1 unit across the mitochondrial membrane), thymidylate synthase (nucleotide synthesis) or folyl poly-γ-glutamate synthase (metabolism of dietary folates). Could such genes provide information as to the specificity of folate in the one-carbon pathways in relation to choline?

(6) The exact role of the two binding proteins, transcobalamin and haptocorrin, have in the transport of cobalamins in the plasma, membrane receptors and tissues is not known. This is also the case for the metabolic activation of vitamin B6 by pyridoxal kinase and pyridoxal phosphate oxidases would benefit from a genetic approach while PLP-dependent enzymes such as δ-aminolevulinate synthase or transaminases would target specific activities of the vitamin. Are transgenic methods applicable in the study of the metabolism of cobalamin and vitamin B6?

(7) Some of the current knock-out models are homozygous lethal, whether at the embryonic [118,121] or post-natal [119,120] stage, which greatly impairs the
use of these models for investigations of tissue specific effects or age-related deficiencies. Could other genetic models such as tissue targeted gene silencing or conditional knock-out that can be activated at later stages provide information in these cases?

Finally, it is evident that deficiencies in B vitamin intakes could be responsible for a wide range of diseases including those of one-carbon transfer units (Table 2). Prevention and treatment of these conditions will undoubtedly be significantly advanced with a further understanding of the complex mechanisms involved. What are the most robust and reliable markers of marginal B vitamin diets that could be used to evaluate vitamin status in case-control, cohort and clinical intervention studies?

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